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Notch Activation by the Metalloproteinase ADAM17 Regulates Myeloproliferation and Atopic Barrier Immunity by Suppressing Epithelial Cytokine Synthesis

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SUMMARY

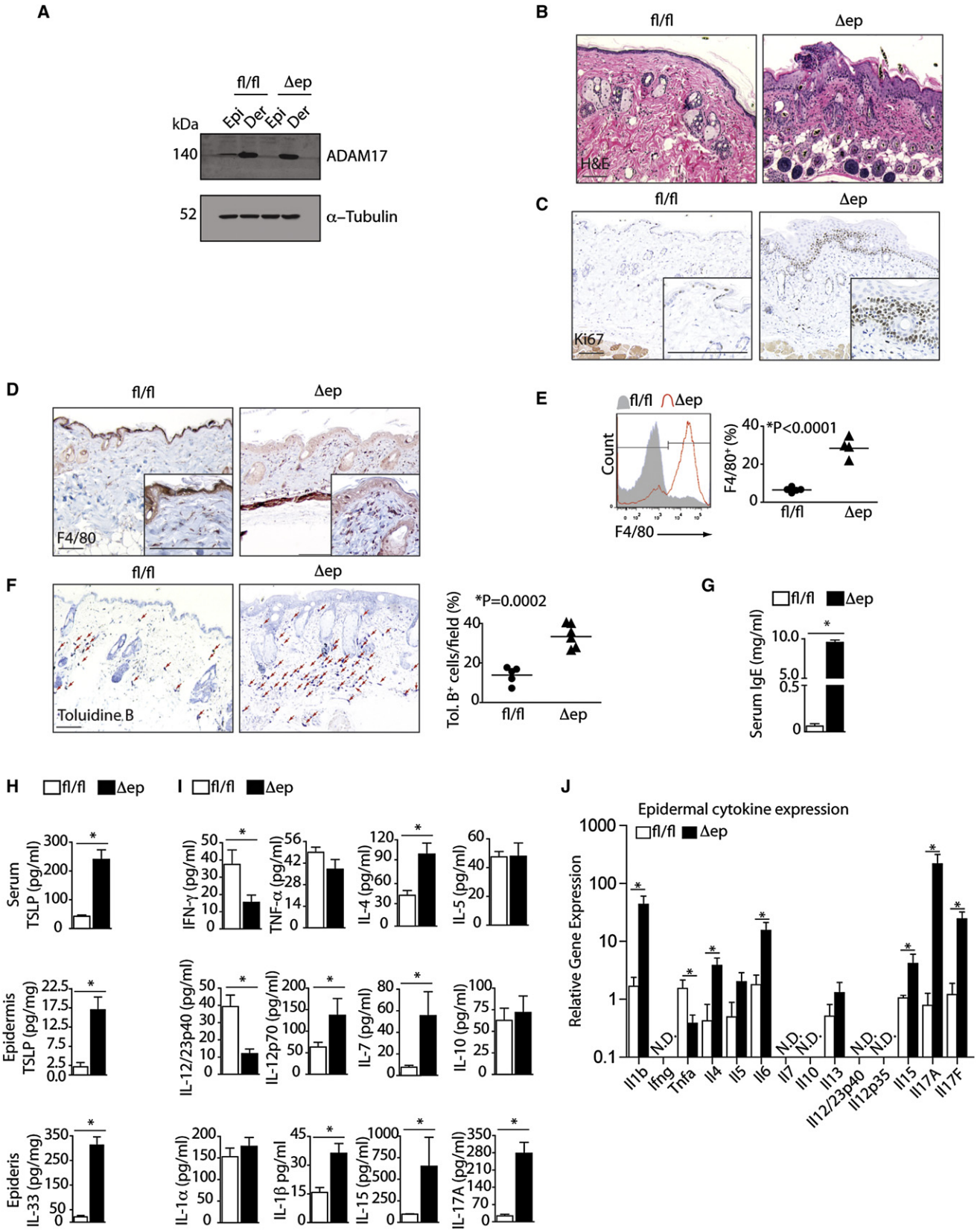
Epithelial cells of mucosal tissues provide a barrier against environmental stress, and keratinocytes are key decision makers for immune cell function in the skin. Currently, epithelial signaling networks that instruct barrier immunity remain uncharacterized. Here we have shown that keratinocyte-specific deletion of a disintegrin and metalloproteinase 17 (*Adam17*) triggers T helper 2 and/or T helper 17 (Th2 and/or Th17) cell-driven atopic dermatitis and myeloproliferative disease. In vivo and in vitro deficiency of ADAM17 dampened Notch signaling, increasing production of the Th2 cell-polarizing cytokine TSLP and myeloid growth factor G-CSF. Ligand-independent Notch activation was identified as a regulator of AP-1 transcriptional activity, with Notch antagonizing c-Fos recruitment to the promoters of *Tslp* and *Csf3* (G-CSF). Further, skin inflammation was rescued and myeloproliferation ameliorated by delivery of active Notch to *Adam17*$^{-/-}$ epidermis. Our findings uncover an essential role of ADAM17 in the adult epidermis, demonstrating a gatekeeper function of the ADAM17-Notch-c-Fos triad in barrier immunity.

INTRODUCTION

Inflammatory skin diseases afflict more than 10% of people in the western world (Bieber, 2008). Keratinocytes are the predominant epithelial cell type that generates the epidermal layer of the skin. The onset of epidermal inflammation represents a collapse of epithelial homeostasis resulting in the generation of alarm signals by keratinocytes. This in turn recruits the immune system to eliminate infected and/or damaged cells and restore homeostasis. Factors produced by epithelial cells that guide immune cell functions are being recognized as the "epimmunome" and it is important to elucidate the mechanisms that underpin their synthesis. Stress response pathways (e.g., NF-κB, AP-1) have emerged as key junctions in the maintenance of epithelial cell homeostasis (Swamy et al., 2010). Disruption of either developmental programming or induction of physiological stress in keratinocytes that compromises barrier function leads to copious production of alarmins including thymic stromal lymphoprotein (TSLP) and interleukin 33 (IL-33), as well as hematopoietic growth factors such as granulocyte colony stimulating factor (G-CSF) or granulocyte macrophage colony stimulating factor (GM-CSF). Over time these generate atopic dermatitis by TSLP-mediated activation of dermal dendritic cells, which results in a local cellular immune response (Demehri et al., 2009; Ziegler and Artis, 2010). Additionally, increased production of myeloid growth factors by keratinocytes has been shown to be causal to the development of myeloproliferative disease (MPD) in mouse models of inflammatory skin disease (Dumortier et al., 2010; Meixner et al., 2008). Therefore, keratinocytes in the epidermis direct local immunity and hematopoietic development, yet epithelial signaling networks that instruct the epimmunome are poorly defined.

The Notch pathway is a master regulator of cell fate decisions and has established roles in directing differentiation of all cell layers in metazoans. Recent emerging evidence also supports a role for this pathway in the maintenance of adult tissue homeostasis. In epithelial tissues such as the skin, Notch signaling sustains the epidermal barrier by promoting cell-autonomous keratinocyte terminal differentiation (Blanpain et al., 2006). Importantly, it also orchestrates communication between the epithelial and immune compartments of the skin to prevent chronic inflammation. For example, recent studies of Notch in adult tissue homeostasis have highlighted its requirement in preventing inflammatory skin disease (Dumortier et al., 2010; Goud and Deshpande, 2011); however, the molecular mechanisms by which epithelial Notch performs this immunoregulatory function remain elusive.

A disintegrin and metalloproteinase 17 (ADAM17), also called TNF alpha converting enzyme (TACE), is a transmembrane metalloproteinase that cleaves cell surface proteins (Blobel, 2005; Murphy et al., 2008). It is essential for postnatal survival in mice and is implicated in immune cell development and function (Horiuchi et al., 2007; Li et al., 2007a, 2007b). ADAM-mediated shedding of Notch receptors (termed S2 cleavage) is a key step in activating Notch signaling, but to date only ADAM10 has been shown to perform this action in vivo, as demonstrated by its role in marginal zone B cell development (Gibb et al., 2010). ADAM10 also performs S2 cleavage of Notch in keratinocytes, and epidermal ADAM10 deficiency leads to


A
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D
fl/fl
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F4/80
E
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Count
F4/80
F4/80+ (%)
*P<0.0001
fl/fl
Δep
F
fl/fl
Δep
Toluidine B
Tol. B+ cells/field (%)
*P=0.0002
fl/fl
Δep
G
fl/fl
Δep
Serum IgE (mg/ml)
H
fl/fl
Δep
Serum TSLP (pg/ml)
Epidermis TSLP (pg/mg)
Epideris IL-33 (pg/mg)
I
fl/fl
Δep
IFN-γ (pg/ml)
TNF-α (pg/ml)
IL-4 (pg/ml)
IL-5 (pg/ml)
IL-12/23p40 (pg/ml)
IL-12p70 (pg/ml)
IL-7 (pg/ml)
IL-10 (pg/ml)
IL-1α (pg/ml)
IL-1β pg/ml
IL-15 (pg/ml)
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Epidermal cytokine expression
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Relative Gene Expression
N.D.
Il1b
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Il7
Il10
Il13
Il12/23p40
Il12p35
Il15
Il17A
Il17F

defective barrier formation (Weber et al., 2011). However, the relevance of ADAM17 in epithelial:immune crosstalk is unknown. We investigated this by inactivating *Adam17* in the epidermis of adult mice. The main outcomes were spontaneous onset of atopic dermatitis (AD) and myeloproliferative disease (MPD). Keratinocyte-specific deletion of *Adam17* triggered spontaneous peripheral accumulation of T helper 2 (Th2), Th17, and γδ T cells, dermal mast cell infiltration, and elevated serum IgE. In parallel, an increase in Gr-1$^+$CD11b$^+$ cells and concomitant loss of B220$^+$ B lymphocytes was observed in the bone marrow. By using in vivo and in vitro approaches, we found that ADAM17 is crucial for basal Notch activation in adult epidermis and that ectopic Notch activation was sufficient to rescue local skin inflammation and MPD in mutant mice. We further identified elevated activator protein-1 (AP-1) transcriptional activity as causal to TSLP and G-CSF production. Analysis of signal crosstalk between Notch and AP-1 pathways in mouse and human keratinocytes revealed that Notch antagonized c-Fos recruitment to epithelial cytokine promoters to keep stress signaling in check. Collectively, our results demonstrate that ADAM17 permits tonic Notch activation in the adult epidermis to regulate epithelial cytokine production and maintain barrier immunity.

RESULTS

Onset of Spontaneous Atopic Dermatitis upon Deletion of Epidermal *Adam17*

Keratinocytes comprise the vast cellular majority of the epidermis. Basal keratinocytes reside at the epidermal-dermal interface and migrate outward toward the skin surface as they undergo terminal differentiation. We induced *Adam17* deletion in basal keratinocytes by breeding *Adam17*$^{fl/fl}$ and Krt14-*cre* transgenic mice expressing the *cre* recombinase under control of the *Keratin14* promoter (Figure 1A). These mice (termed *Adam17*$^{\Delta ep}$) exhibited a progressive atopic-dermatitis-like inflammatory skin disease with 100% penetrance as early as 3 weeks of age. Hematoxylin and eosin staining showed epidermal thickening, a loss of subcutaneous fat, and regions of hyperkeratosis, and immunohistochemical analysis with the proliferation marker Ki67 revealed keratinocyte hyperproliferation (Figures 1B and 1C). Histological and FACS analysis revealed massive infiltration by F4/80$^+$ macrophages (Figures 1D and 1E; FACS quantification in scatterplot to the right) and Toluidine-B$^+$ mast cells in the dermis (arrows, Figure 1F; quantification in scatterplot to the right). Consistent with mast cell influx, serum IgE concentrations were >100-fold higher in *Adam17*$^{\Delta ep}$ mice (Figure 1G). We also measured a marked increase in thymic stromal lymphoprotein (TSLP) and interleukin-33 (IL-33), both in the epidermis and systemically (Figure 1H). These data indicated spontaneous onset of AD in mice lacking epidermal ADAM17.

The production of Th2 cell cytokines typifies atopic diseases (Fort et al., 2001). We observed dramatic changes in serum concentrations of specific factors, most notably the elevation of Th2 (IL-4, IL-15) and reduction in Th1 (IFN-γ, IL-12p40) cell cytokines in 10-week-old mutant mice (Figure 1I). In addition to Th2 cell cytokine production, cutaneous mechanical injury caused by dry, itchy skin is a major contributor to the generation of IL-17-producing lymphocytes (Jin et al., 2009). The accumulation of IL-17-producing T lymphocytes in peripheral blood also correlates significantly with disease severity of AD in human patients (Di Cesare et al., 2008; Koga et al., 2008). Mutant mice exhibited greater serum concentrations of IL-17A and its polarizing cytokine IL-6 (Ogura et al., 2008) (Figure 1I). Direct gene expression analysis of the epidermis showed significantly higher expression of inflammatory cytokines IL-1β, IL-4, IL-6, IL-15, and IL-17A and IL-17F in mutant skin. Of note, epidermal TNFα expression was decreased in ADAM17-deficient skin, and both IL-12 subunits (IL-12p40 and IL-12p35) were not detected (Figure 1J). Thus, epidermal *Adam17* loss results in a spontaneous Th2 or Th17 cell-dominant atopic immune response.

Th2 and Th17 Cell Polarized Cellular Immunity in Skin-Draining Lymph Nodes of *Adam17*$^{\Delta ep}$ Mice

In order to characterize the nature and origin of the cellular immune response, we compared skin-draining (dLN) and mesenteric (mLN) lymph nodes. Along with obvious lymphadenopathy (data not shown), FACS analysis showed a 10-fold elevation in Gr-1$^+$CD11b$^+$ monocytes and the presence of CD11c$^+$MHCII$^+$ antigen-presenting cells (APCs) in *Adam17*$^{\Delta ep}$ mice (Figures 2A and 2B). Although both CD8$^+$ and CD4$^+$ T lymphocytes were activated in the dLNs of *Adam17*$^{\Delta ep}$ mice (histograms, Figure 2C), CD4$^+$ T cell activation was induced to a much greater magnitude as indicated by the 8-fold higher numbers of CD4$^+$CD69$^+$ versus CD8$^+$CD69$^+$ T cells (bar graphs, Figure 2C). Lymphocyte activation follows a shift from naive (CD62L$^+$CD44$^-$) to effector (CD62L$^-$CD44$^+$) T cell phenotypes (Fang et al., 2002). *Adam17*$^{\Delta ep}$ mice had a 4-fold increase in CD4$^+$ effector T cells specifically in dLN but not in mLN (Figure 2D). Next, FACS analysis of phorbol myristate acetate (PMA)-stimulated lymphocytes obtained from skin-draining lymph nodes showed a measurable number of CD4$^+$IL-17A$^+$ Th17 cells (Figure 2E) and a large population of γδTCR$^+$IL-17A$^+$ γδT cells in the dLNs of *Adam17*$^{\Delta ep}$ mice (Figure 2F). These data confirm the skin-specific origin of cellular immunity in

Figure 1. Epidermal ADAM17 Deficiency Causes Atopic Dermatitis

(A) ADAM17 immunoblot performed on tail epidermis and dermis of adult *Adam17*$^{fl/fl}$ and *Adam17*$^{\Delta ep}$ mice.
(B and C) Histological analysis performed on dorsal skin (B, H&E) and keratinocyte proliferation (C, Ki67 immunostaining).
(D and E) Immunohistochemical and flow cytometry (FACS) quantification for F4/80 to measure macrophage infiltration in dermis.
(F) Toluidine-B staining performed to measure mast cell infiltration (red arrows), quantified on the right.
(G) Serum IgE measured by ELISA.
(H) Serum and tail epidermis analyzed by ELISA for concentrations of TSLP and IL-33.
(I) Bead array profiling of serum for indicated cytokines.
(J) qPCR analysis of indicated cytokine mRNAs from tail epidermis of *Adam17*$^{fl/fl}$ and *Adam17*$^{\Delta ep}$ mice.
*$p < 0.05$ mean ± SEM ($n \geq 4$). Histology and immunoblotting data are representative of at least six mice. Scale bars represent 100 μm.

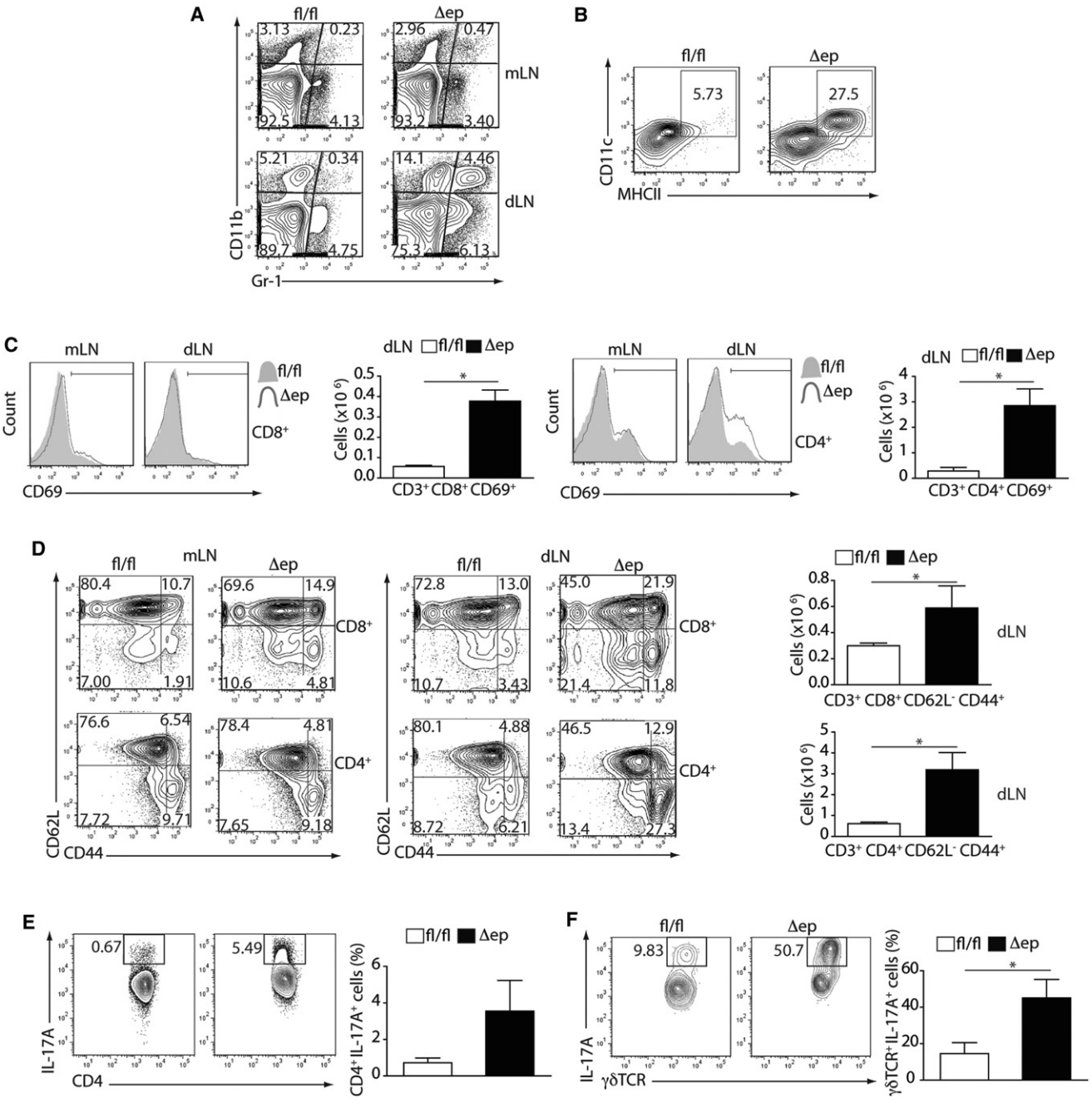

Figure 2. Skin-Specific Lymphocyte Activation and IL-17A Production in *Adam17*$^{\Delta ep}$ Mice

(A) FACS analysis of macrophage-monocytes (Gr-1$^+$CD11b$^+$) in skin-draining lymph nodes (dLN) and mesenteric lymph nodes (mLN).

(B) FACS analysis of activated (CD11c$^+$MHCII$^+$) dendritic cells in dLNs.

(C) FACS analysis of CD8$^+$ versus CD4$^+$ T cell activation by CD69 staining in mLNs and dLNs. Bar graphs are quantifications of histograms.

(D) Induction of CD4$^+$ effector T cells (CD62L$^-$CD44$^+$) in dLNs of Δep mice measured by FACS. Bar graphs are quantifications of indicated populations shown in representative plots.

(E) Identification of γδTCR$^+$IL-17A$^+$ populations in dLN lymphocytes restimulated ex vivo with PMA+ionomycin in the presence of Brefeldin A. Bar graphs indicate relative abundance of IL-17A-producing cells.

(F) Analysis of CD3$^+$CD4$^+$IL-17A$^+$ (Th17) cell populations, dLN lymphocytes restimulated as in (E). Bar graph depicts relative abundance of CD3$^+$CD4$^+$IL-17A$^+$ cells.

*$p < 0.05$ mean ± SEM. Data in (A)–(D) are representative of at least 12 mice. Data in (F) and (G) are representative of 7 mice.

mice lacking epidermal ADAM17 and reveal peripheral γδ and Th17 cells as the source of IL-17A.

Onset of Severe Myeloproliferative Disease in *Adam17*$^{\Delta ep}$ Mice

Mice harboring genetic epidermal defects often exhibit myeloproliferative disease (MPD) (Dumortier et al., 2010; Meixner et al., 2008). Our investigation of *Adam17*$^{\Delta ep}$ mice revealed strikingly high concentrations of serum G-CSF along with the presence of splenomegaly (Figure S1A available online and data not shown). Interestingly, we noted a severe drop in bone marrow B220^{+} lymphocyte populations in *Adam17*$^{\Delta ep}$ mice, which upon further characterization were found to be predominantly naive B lymphocytes expressing high amounts of IgM compared to controls (Figures S1B and S1C). Although the total number of bone marrow cells were comparable (data not shown), Gr-1^{+}CD11b^{+} myeloid populations were proportionally higher in bone marrow and spleen of *Adam17*$^{\Delta ep}$ mice (Figure S1D), fulfilling all of the hallmarks of MPD (Campbell and Green, 2006). We postulated that this myeloid cell expansion contributed to higher IL-12p70 concentrations observed in the serum of *Adam17*$^{\Delta ep}$ mice (Figure 1I) and assayed the capacity of spleen and bone marrow cells to produce IL-12p70 or IL-12p40. Stimulating total spleen and bone marrow cells ex vivo with LPS in the presence of IFN-γ resulted in markedly greater IL-12p70 production by cells obtained from *Adam17*$^{\Delta ep}$ mice (Figure S1E). IL-12p40 concentrations were lower in the serum and in unstimulated (basal) cultures of spleen cells obtained from *Adam17*$^{\Delta ep}$ mice (Figures 1J and S2G). LPS+IFN-γ stimulation induced IL-12p40 production ex vivo, indicating that epidermal ADAM17 deficiency did not compromise cell-autonomous capacity to produce IL-12p40 (Figure S1F). These data demonstrate that epidermal ADAM17 deficiency is sufficient to induce the expansion of IL-12p70-producing myeloid cells in mice.

TNFR1 Signaling Is Dispensable for Atopic Dermatitis and Myeloproliferative Disease in *Adam17*$^{\Delta ep}$ Mice

TNF inhibitors have had measurable success in therapy of inflammatory skin disease in humans (Guinea-Viniegra et al., 2009; Leung et al., 2004; Pasparakis et al., 2002). Independent genetic models show that psoriasis-like inflammatory skin disease caused by epidermal loss of *Ikkb* or *Jun* and *Junb* is ameliorated by the deletion of TNF receptor 1 (*Tnfr1*), but the importance of TNFR1 signaling in models of atopic dermatitis has not been tested. ADAM17 is the most relevant sheddase for the cleavage of membrane-bound TNF and its receptors, and we have previously shown that regulation of ADAM17 activity by its endogenous inhibitor TIMP3 provides a critical axis of control over TNF in models of acute and chronic hepatic inflammation (Mohammed et al., 2004; Murthy et al., 2010; Smookler et al., 2006). Therefore, we bred *Adam17*$^{\Delta ep}$ mice into a *Tnfr1*-deficient background and surprisingly found that *Tnfr1* loss failed to rescue epidermal inflammation. Analyses of peripheral immunity demonstrated activation of CD4^{+} T cells (Figure S2A), induction of CD4^{+} effector T cells (Figure S2B), and elevated major histocompatibility complex class II (MHCII) expression on APCs in dLN of *Adam17*$^{\Delta ep}$*Tnfr1*$^{-/-}$ mice (Figure S3C). *Adam17*$^{\Delta ep}$*Tnfr1*$^{-/-}$ mice also exhibited MPD, with increased proportions of Gr-1^{+}CD11b^{+} myeloid cells and a loss of B220^{+} B lymphocytes in the bone marrow (Figures S2D and S2E). Thus, epidermal ADAM17 prevents atopic dermatitis and maintains bone marrow homeostasis independent of TNFR1 signaling.

Precocious Differentiation of ADAM17-Deficient Keratinocytes In Vivo and In Vitro

ADAMs perform ectodomain shedding of several factors required for keratinocyte development including epidermal growth factor receptor (EGFR) ligands and Notch. For example ADAM17 cleaves a subset of EGFR ligands whereas ADAM10 is the recognized metalloproteinase for Notch processing. The epidermal loss of *Adam10* was recently reported to impair keratinocyte differentiation in mice; they failed to establish the spinous layer of the epidermis (Weber et al., 2011). We therefore asked whether the observed epidermal inflammation arose because of incomplete keratinocyte differentiation. This was not the case for *Adam17*$^{\Delta ep}$ mice; immunofluorescence staining of the skin showed comparable establishment of terminal keratinocyte differentiation measured by basal (Keratin 5), early (Keratin10), and late (Filaggrin) markers (Figure 3A). Further, gene expression analysis confirmed the presence of basal and spinous (early) lineages along with increased expression of granular (late) lineage markers in the epidermis of newborn *Adam17*$^{\Delta ep}$ mice (Figure 3B). We next created cell-autonomous ex vivo deletion of *Adam17* with *Adam17*$^{fl/fl}$ keratinocytes transduced with adenoviral Cre (termed AdCre-*Adam17*$^{-/-}$), and we tested the differentiation capacity of these cells by using an established method of inducing terminal differentiation, i.e., increasing extracellular concentrations of calcium to >1.0 mM. AdCre-*Adam17*$^{-/-}$ keratinocytes exhibited accelerated differentiation as measured by gene expression of basal, early (spinous), and late (granular or cornified) markers (Figure 3C). Of note, adenoviral deletion of *Adam17* induced precocious keratinocyte differentiation even in basal conditions (AdCre Low Ca^{2+} group, Figure 3C). Consistent with the onset of terminal differentiation, AdCre-*Adam17*$^{-/-}$ keratinocytes proliferated at a slower rate in basal conditions when compared with control (AdGFP) keratinocytes (Figure 3D). These data indicate that in contrast to ADAM10 deficiency, loss of *Adam17* leads to precocious keratinocyte differentiation in a cell-autonomous manner.

Inducible Deletion of Epidermal *Adam17* in Adult Mice Recapitulates Atopic Dermatitis and Myeloproliferative Disease

Beyond keratinocyte differentiation, we reasoned that ADAM17 would be required for epidermal homeostasis in the adult skin. We therefore bred *Adam17*$^{fl/fl}$ mice with transgenic mice expressing tamoxifen-inducible *cre*-recombinase under control of the *Krt14* promoter (termed *Adam17*$^{\Delta epERT}$ mice) and induced *Adam17* deletion as outlined in Figure 4A. Mice were analyzed at early (3 month) and late (8 month) time points postdeletion. Tamoxifen-induced deletion of *Adam17* resulted in lymphadenopathy (data not shown) and skin inflammation. We observed epidermal thickening (H&E, Figure 4B) and keratinocyte hyperproliferation as measured by Ki67 staining as early as 3 months

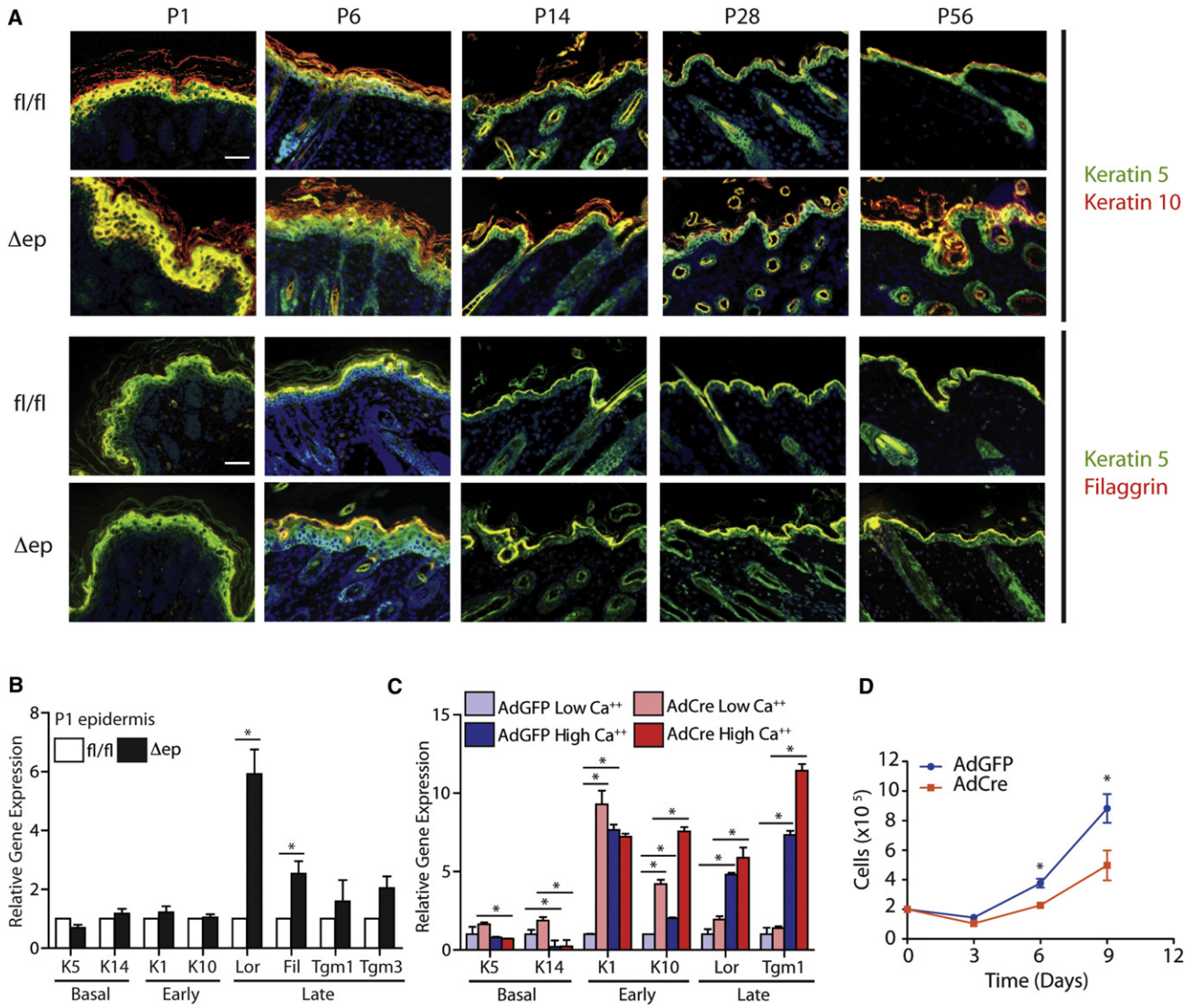

Figure 3. Precocious Differentiation in *Adam17*$^{\Delta ep}$ Epidermis

(A) Histological (immunofluorescence) analysis of skin at indicated postnatal ages (P) measuring expression of basal marker Keratin5 (K5, green), early differentiation marker Keratin10 (K10, red), and late differentiation marker Filaggrin (Fil, red).

(B) qPCR analysis of basal, early, and late differentiation markers in newborn mice (postnatal day1, P1).

(C) qPCR analysis of keratinocyte differentiation markers in primary keratinocytes after calcium-induced terminal differentiation. Cells were maintained in basal medium (low Ca^{2+}) or treated with 2 mM calcium (high Ca^{2+}) for 48 hr. AdGFP (control): *Adam17*$^{fl/fl}$ keratinocytes transduced with adenoviral-GFP; AdCre (*Adam17* deleted): *Adam17*$^{fl/fl}$ keratinocytes transduced with adenoviral-Cre to induce cell-autonomous *Adam17* deletion.

(D) Decreased rate of proliferation in AdCre-*Adam17*$^{-/-}$ keratinocytes cultured in basal conditions.

*$p < 0.05$, mean ± SEM (n = 4 for B, n = 3 for C and D). Images are representative of at least four mice per genotype. Scale bars represent 50 mm. All data are representative of two independent experiments.

(Figure 4C) as well as dermal infiltration by Toluidine B^+ mast cells (arrows, Figure 4D).

FACS analysis indicated higher numbers of $CD4^+CD69^+$ activated T cells in dLNs of *Adam17*$^{\Delta epERT}$ mice (Figure 4E, quantified in Figure 4F). Additionally, a significantly higher proportion of $CD4^+CD62L^-CD44^+$ effector T cells was observed in dLNs of *Adam17*$^{\Delta epERT}$ mice at all ages (Figure 4G, quantified in Figure 4H). Finally, these mice developed MPD as characterized by loss of $B220^+$ B lymphocytes (Figures S3A and S3B) and proportionally greater populations of Gr-1^+CD11b^+ macrophage-monocytes in bone marrow and spleen (Figures S3C and S3D). These experiments establish that deletion of epidermal *Adam17* in the adult skin compromises barrier immunity and results in myeloproliferative disease.

Loss of *Adam17* Compromises Tonic Notch Signaling in the Epidermis

Remarkably, *Adam17*$^{\Delta ep}$ mice closely phenocopy the onset of AD and MPD previously illustrated upon inducible and combined postnatal deletion of epidermal *Notch1* and *Notch2* (Dumortier

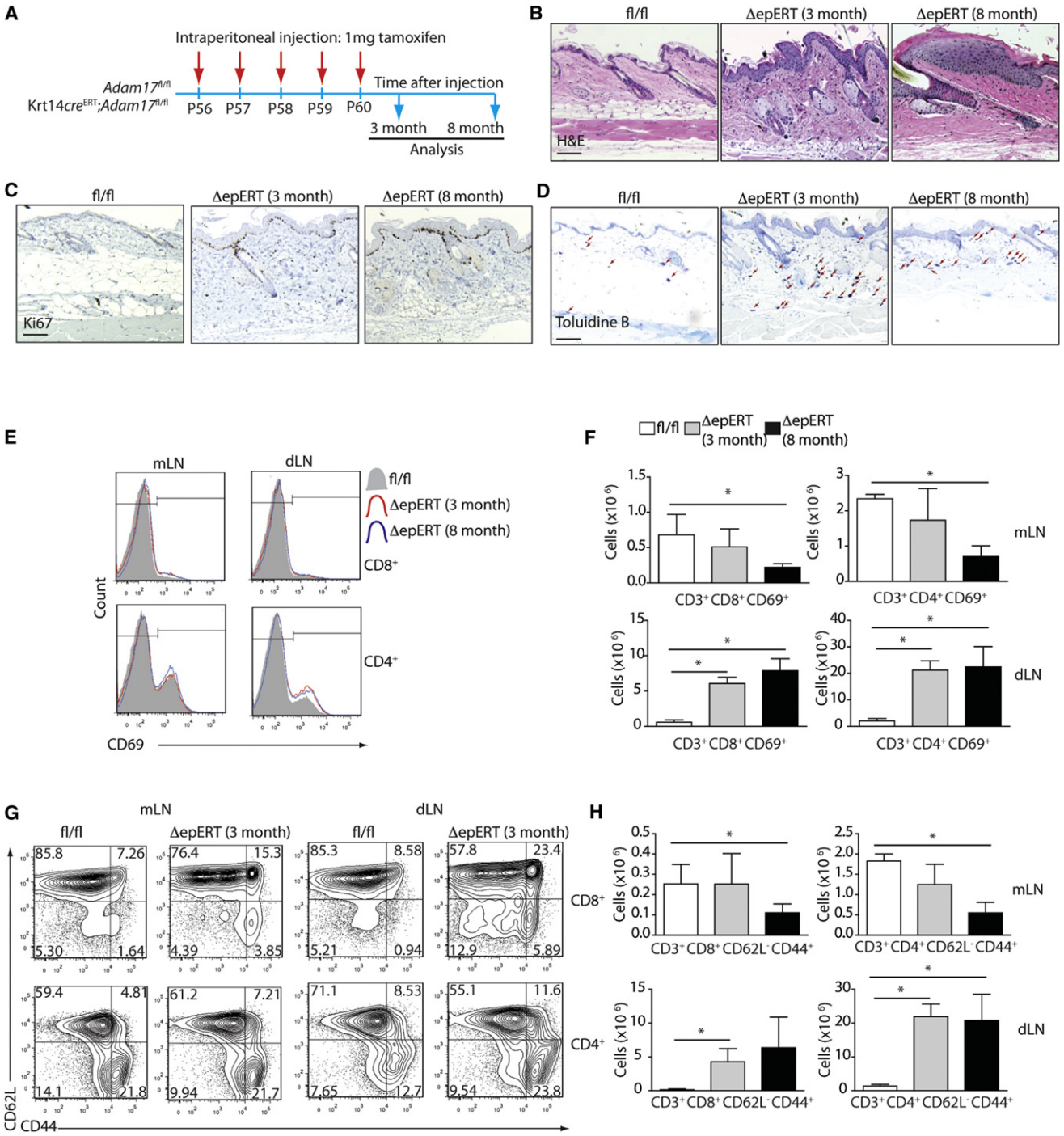

Figure 4. Inducible Deletion of Epidermal *Adam17* in Adult Mice Recapitulates Atopic Dermatitis and Skin-Specific CD4$^+$ Lymphocyte Activation

(A) Schematic depicting protocol for tamoxifen treatment and mouse analysis.

(B–D) Histological analysis of dorsal skin by H&E staining (B) Ki67 immunostaining depicting basal keratinocyte proliferation (C) and mast cell influx (D, arrows) shown by Toluidine B staining.

(E and F) FACS plots and quantification of CD8$^+$ and CD4$^+$ T cell activation in mesenteric lymph nodes (mLN) and skin-draining lymph nodes (dLN) measured by cell surface CD69.

(G and H) FACS plots and quantification of CD8$^+$ and CD4$^+$ effector T cells identified as CD62L$^-$CD44$^+$ populations in mLNs and dLNs of *Adam17*$^{fl/fl}$ and *Adam17*$^{\Delta epERT}$ mice.

*$p < 0.05$, mean ± SEM (n = 4).

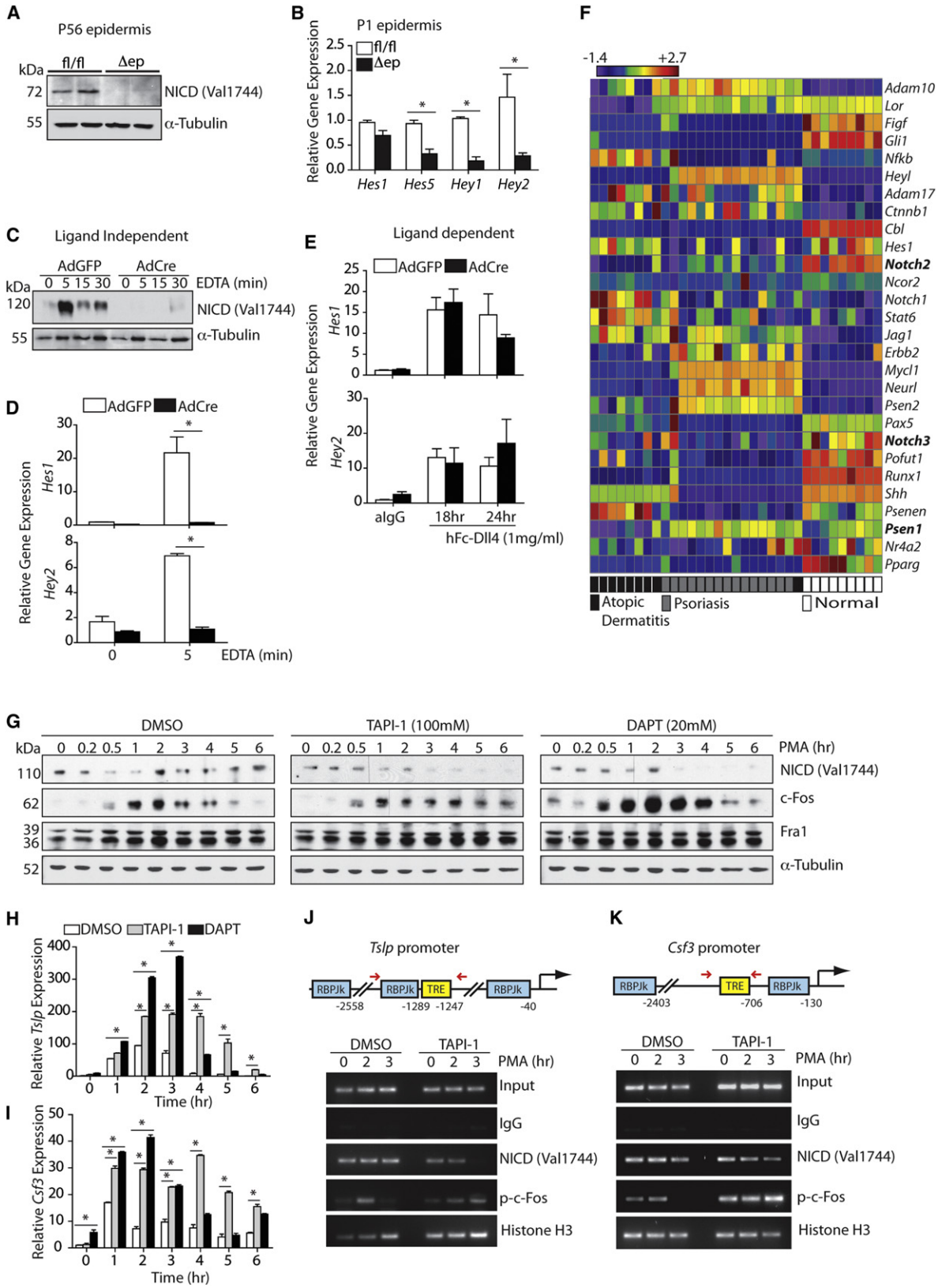

Figure 5. ADAM17 Provides Ligand-Independent Notch Signaling and Suppresses c-Fos Activity in Keratinocytes

(A) Immunoblot depicting loss of NICD production in adult Δep epidermis.

(B) qPCR analysis of newborn (P1) epidermis for Notch target genes *Hes1*, *Hes5*, *Hey1*, and *Hey2*.

et al., 2010). However, there is no physiological evidence for ADAM17-mediated Notch activation in mammals. We tested whether ADAM17-deficient skin had defective Notch signaling and found Notch intracellular domain (NICD) to be absent in adult *Adam17*$^{\Delta ep}$ epidermis (Figure 5A). Moreover, the expression of multiple Notch target genes was diminished in *Adam17*$^{\Delta ep}$ epidermis (Figure 5B). Typically, Notch activation follows ligand binding (Delta-like 1, 3, 4, Jagged 1, 2) and involves metalloproteinase (ADAM)-mediated S2 cleavage of the receptor at the cell surface with subsequent NICD release via γ-secretase (S3 cleavage) (Artavanis-Tsakonas et al., 1999). Notch activation can occur in a ligand-dependent or ligand-independent manner, and different groups have shown that ADAM10, but not ADAM17, is required for ligand-dependent Notch activation in vitro (Bozkulak and Weinmaster, 2009; Gibb et al., 2010). To delineate how ADAM17 contributed to Notch activation in keratinocytes, we examined both possible scenarios for Notch activation by using AdCre-*Adam17*$^{-/-}$ keratinocytes. In a ligand-independent model of Notch activation introduced by Rand et al. (2000), ethylene diamine tetraacetic acid (EDTA) treatment of AdCre-*Adam17*$^{-/-}$ keratinocytes failed to produce NICD or induce Notch target gene expression (Figures 5C and 5D). We next measured specific generation of the ADAM-dependent S2 fragment of Notch by adding the γ-secretase inhibitor DAPT in EDTA-treated keratinocyte cultures. Control (AdGFP) keratinocytes were able to produce both S2 and S3 fragments of Notch after EDTA treatment, and presence of DAPT ablated S3 but not S2 cleavage (Figure S4A). Consistent with the stepwise requirement of S2 Notch cleavage to generate NICD, AdCre-*Adam17*$^{-/-}$ keratinocytes failed to produce either fragment (Figure S4A). Notch signaling in keratinocytes can also be induced by increased calcium exposure (Blanpain et al., 2006). Prolonged treatment with ≥1 mM calcium resulted in NICD generation and Notch signaling by control (AdGFP) keratinocytes, but AdCre-*Adam17*$^{-/-}$ cells were defective in this model of Notch signaling as well (Figures S4B and S4C). On the other hand, ADAM17 was dispensable for ligand-dependent Notch signaling when induced by Fc-Delta-like 4 fusion protein (Figure 5E). Decreased gene expression of *Adam10*, Notch receptors, or the presenilin γ-secretases could contribute to the dampened Notch activation observed in *Adam17*$^{\Delta ep}$ mice. Quantitative polymerase chain reaction (qPCR) analysis of the epidermis showed comparable transcript amounts of Adam9, 10, 12, Notch1, 2, 3, 4, or Psen1 and 2 (Figure S4D). Together, these data demonstrate that ADAM17 activity is required for Notch signaling in adult skin and suggest that ADAM17 activates Notch in a ligand-independent manner in keratinocytes.

We further sought evidence for compromised Notch signaling in patients with inflammatory skin disease by using published microarray human gene expression data sets (Guttman-Yassky et al., 2009; Nair et al., 2009; Olsson et al., 2006; Reischl et al., 2007). We consistently observed genes in the Notch pathway to be differentially expressed in patients with AD, atopic eczema, and psoriasis (Figure 5F and data not shown). Specifically, a decrease in Notch2, Notch3, and Presenilin1 mRNA in AD was observed compared to normal skin (Figure 5F; gene lists in Table S1). Of note, Adam10 and Adam17 mRNA displayed a heterogeneous expression pattern in AD skin, probably because of the pleiotropic functions of these metalloproteinases. We also performed unsupervised clustering analysis and observed that genes in the Notch signaling module stratified AD samples away from psoriasis and normal samples, suggesting that Notch signaling is more deregulated in AD than in psoriasis (Figure S4E). The above data propose a link between ADAM17 and Notch signaling in human atopic skin disease.

Active Notch Antagonizes c-Fos-Mediated Transcription of TSLP in Human Keratinocytes

Our next goal was to identify the mechanism by which ADAM17-driven Notch activation keeps epithelial inflammation in check. Epithelial cytokines TSLP and IL-33 have emerged as key alarmins in barrier immunity (Demehri et al., 2009; Saenz et al., 2008; Ziegler and Artis, 2010). Keratinocytes also produce large amounts of hematopoietic growth factors CSF-3 (G-CSF) and CSF-2 (GM-CSF) in an AP-1-regulated manner (Eferl and Wagner, 2003; Meixner et al., 2008). The AP-1 family has pleiotropic functions in stress response where specific members dimerize, bind to target sequences, and regulate gene expression (Eferl and Wagner, 2003). Meanwhile, Notch has been proposed to inhibit AP-1 activity in vitro (Chu et al., 2002; Talora et al., 2002). To interrogate members of the AP-1 transcription factor family antagonized by Notch, we used primary mouse keratinocytes and a human keratinocyte cell line (HaCaT cells). We began by measuring individual AP-1 proteins (c-Jun, JunB, JunD, c-Fos, FRA1, FRA2) in HaCaT cells after treatment with the phorbol ester PMA (an AP-1 agonist) over 6 hr. Of these AP-1 members, c-Fos displayed a distinct profile with its amounts peaking at 2 hr and rapidly terminating at 3 hr (Figure S5A). c-Fos typically dimerizes with the Jun proteins (c-Jun, JunB,

(C) Immunoblot depicting a lack of S3 cleavage in AdCre-*Adam17*$^{-/-}$ keratinocytes after 5 mM EDTA treatment for the indicated times.
(D) qPCR measurement of Notch target gene expression (*Hes1*, *Hey2*) induced by EDTA treatment for 5 min followed by recovery for 4 hr.
(E) qPCR of Notch target gene expression in AdGFP-control and AdCre-*Adam17*$^{-/-}$ keratinocytes after ligand-dependent Notch activation by Delta like 4 (hFc-DLL4).
(F) Analysis of published microarray gene expression data sets indicates differential expression of genes involved in Notch signaling in atopic dermatitis and psoriasis patients compared to normal controls.
(G) Immunoblotting for NICD, c-Fos, and FRA1 in HaCaT cells pretreated with ADAM17 inhibitor TAPI-1 or γ-secretase inhibitor DAPT, followed by PMA treatment for the indicated time points.
(H and I) qPCR measurement of TSLP and CSF-3 (G-CSF) gene expression in HaCaT cells treated as in (G).
(J and K) HaCaT cells were pretreated with DMSO (control) or the ADAM17 inhibitor TAPI-1, followed by PMA treatment for the indicated times. Chromatin was precipitated with the indicated antibodies. *Tslp* and *Csf3* promoters were amplified from precipitated DNA. Schematics of human *Tslp* and *Csf3* promoters depict AP-1 binding sites (TRE), Notch binding sites (RBP-jκ), and primer design (red arrows).
*$p < 0.05$ mean ± SEM (n = 4 for B, n = 3 for D, E, H, I). Data in (C)–(E) are representative of three independent experiments; data in (G)–(K) represent two independent experiments.

JunD) (Eferl and Wagner, 2003) and their amounts gradually increased in HaCaT cells after PMA stimulation (Figure S5A). We inhibited either ADAM17 activity or NICD production by using small molecule inhibitors (TAPI-1 and DAPT, respectively), and both resulted in a sustained production of c-Fos upon PMA treatment (Figure 5G). TAPI-1 or DAPT treatment also led to the depletion of NICD as measured by immunoblotting (Figure 5G). Notably, c-Fos stabilization or NICD depletion correlated with elevated TSLP and GM-CSF mRNA transcription as measured by qPCR (Figures 5H and 5I).

The promoter regions of *Tslp* and *Csf3* contain 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (TRE) that bind AP-1, and promoter polymorphisms that enhance AP-1 binding are associated with higher susceptibility to bronchial asthma (Harada et al., 2010). These promoters also harbor RBP-jκ binding sites that recruit the Notch transcriptional complex (schematics for human promoters shown in Figures 5J and 5K, mouse promoters in Figure S5). We assessed c-Fos and NICD recruitment to these promoters by chromatin immunoprecipitation (ChIP) assays. NICD was constitutively present on the *Tslp* promoter in HaCaT cells and primary keratinocytes whereas c-Fos was only recruited at 2 hr after PMA treatment (Figures 5J, 5K, S5B, and S5C). In HaCaT cells, inhibition of ADAM17 activity with TAPI-1 depleted NICD production and abolished its recruitment to the *Tslp* promoter while concomitantly prolonging c-Fos binding to this region at 3 hr after PMA treatment (Figure 5J). Similar but not identical antagonism was observed between NICD and c-Fos at the *Csf3* promoter (Figure 5K). Next, ChIP analysis of mouse *Tslp* and *Csf3* promoters was performed by qPCR amplification. AdCre-*Adam17*$^{-/-}$ keratinocytes demonstrated a loss of NICD recruitment to the recombining binding protein suppressor of hairless (RBP-jκ) binding sites of *Tslp* and *Csf3* (Figures S5B and S5C). Conversely, PMA stimulation resulted in a ~3-fold increase of c-Fos recruitment to TRE of *Tslp* and *Csf3* promoters (Figures S5B and S5C). Thus, we show that loss of Notch activation allows for unchecked c-Fos transcriptional activity in human and mouse keratinocytes.

Ectopic Notch Rescues the Enhanced AP-1-Driven Stress Response in ADAM17-Deficient Keratinocytes

We next determined whether tonic Notch activation by ADAM17 keeps AP-1-dependent cytokine production in check. Adenoviral Cre-mediated *Adam17* deletion in keratinocytes (AdCre-*Adam17*$^{-/-}$) resulted in spontaneous phosphorylation of the stress kinase JNK 1 and 2 (Figure 6A). Treatment with PMA specifically enhanced the protein amounts of c-Fos and another AP-1 member FRA1 in AdCre-*Adam17*$^{-/-}$ compared to AdGFP control keratinocytes. Additionally, the DNA binding activity of AP-1 members was elevated in AdCre-*Adam17*$^{-/-}$ keratinocytes (Figures 6B and 6C). Next, qPCR analysis showed higher TSLP, CSF-2, CSF-3, and c-Fos expression in AdCre-*Adam17*$^{-/-}$ keratinocytes after PMA treatment; this was abrogated by the pan AP-1 inhibitor Tanshinone IIA (TanIIA) (Figure 6D, black bars; Figure S6A). We noted the specificity of this response because ADAM17 deficiency did not alter gene expression of either CSF-1 or IL-33 cytokines or the other AP-1 members (c-Jun, JunB, JunD, FRA1, FRA2) (Figure S6A). These data show that the loss of *Adam17* results in increased AP-1-driven synthesis of the above epithelial cytokines.

IL-33 is a nuclear cytokine released upon epithelial stress and is an early activating signal for mast cells. We and others have previously demonstrated that epithelial cell survival and migration depends in part on the availability of growth factors derived from the EGF family (e.g., amphiregulin, TGF-α, HB-EGF); ectodomain shedding by ADAM17 regulates the availability of these ligands (Maretzky et al., 2011; Murthy et al., 2010; Sibilia et al., 2000; Stoll et al., 2010). More recently, genetic loss of *Adam17* in models of colitis has revealed its requirement in providing EGFR ligands to gut epithelial cells. Mice lacking epithelial ADAM17 fail to regenerate gut epithelial cells after injury and consequently succumb to DSS-induced colitis because of a lack of EGFR ligands (Brandl et al., 2010; Chalaris et al., 2010). We reasoned that ADAM17-deficient keratinocytes may have impaired EGFR activation and will exhibit enhanced stress-induced synthesis of IL-33. High concentrations of PMA induced IL-33 transcription, and this was enhanced in AdCre-*Adam17*$^{-/-}$ keratinocytes (Figure S6B). The addition of soluble EGF suppressed IL-33 transcription, whereas pretreatment with the EGFR receptor tyrosine kinase inhibitor Erlotinib enhanced its transcription even in control keratinocytes (Figure S6B). These data suggest that ADAM-mediated ectodomain shedding may suppress synthesis of specific alarmins by providing epithelial growth factors.

We measured AP-1 activity in an independent cell type (mouse embryonic fibroblasts, MEFs) and observed that *Adam17*$^{-/-}$ MEFs had higher basal AP-1 luciferase reporter activity. This was enhanced after PMA treatment and reduced by a c-Fos inhibitor (T-5224) (Figure 6E). More importantly, transduction of active Notch (AdNICD) in AdCre- *Adam17*$^{-/-}$ keratinocytes ablated the expression of CSF-2, CSF-3, and TSLP (Figure 6F, black bars; Figure S6C). In addition, AdNICD abolished both basal and PMA-induced AP-1 luciferase reporter activity in *Adam17*$^{-/-}$ MEFs (Figure 6E). We therefore conclude that ADAM17-dependent Notch activation inhibits AP-1 transcriptional activity, with c-Fos being a relevant AP-1 candidate.

Adenoviral Delivery of Active Notch Rescues Local Cellular Immunity and Myeloproliferation in *Adam17*$^{\Delta ep}$ Mice

Finally, to test whether Notch could protect *Adam17*$^{\Delta ep}$ mice from AD, we utilized a protocol to induce ectopic Notch activity in ADAM17-deficient epidermis (schematic in Figure 7A). Adenoviral delivery of active Notch (AdNICD) into *Adam17*$^{\Delta ep}$ mice showed a measurable increase in Notch target gene expression by qPCR analysis of epidermal scrapings (Figure 7B). Importantly, CD4$^+$ T cell activation was abrogated in dorsal dLNs and a loss of effector CD4$^+$ T cells was observed in AdNICD-treated mice (Figures 7C and 7D). Dorsal delivery of AdNICD also partially rescued MPD, resulting in a decrease in bone marrow Gr-1$^+$CD11b$^+$ cells (Figure 7E). More importantly, a replenishment of B220$^+$ lymphocytes was seen in AdNICD-treated mice (2.93% in AdGFP versus 13.47% in AdNICD, Figure 7F). Thus, active Notch was sufficient to rescue AD as well as MPD in *Adam17*$^{\Delta ep}$ mice. Altogether these data show that ADAM17 activates Notch and this in turn interferes with c-Fos recruitment to the *Tslp* and *Csf3* promoters to

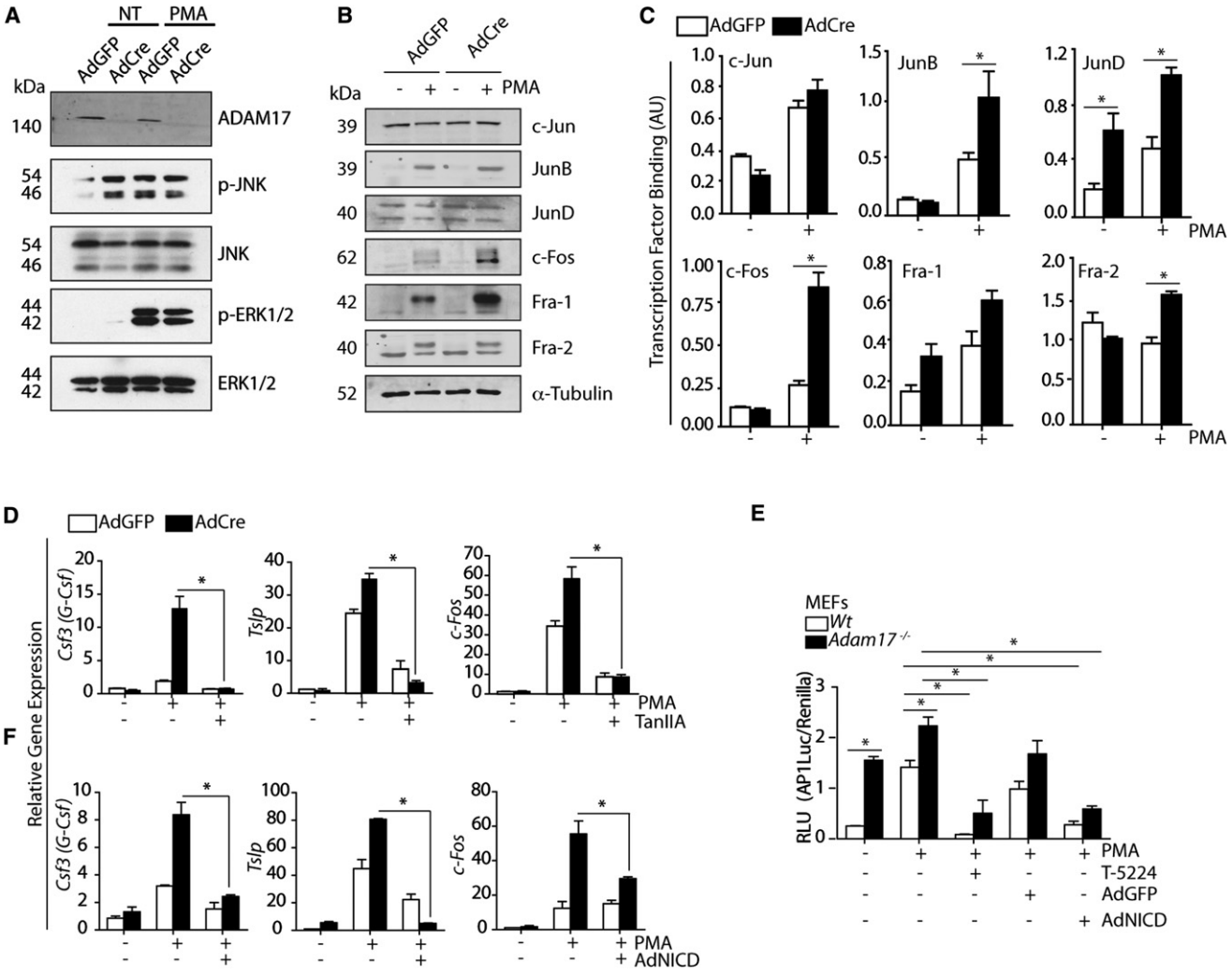

Figure 6. Diminished Notch Activation upon ADAM17 Deficiency Enhances c-Fos-Driven Stress Signaling in Keratinocytes

(A) Immunoblotting detects spontaneous JNK phosphorylation in AdCre-*Adam17*$^{-/-}$ keratinocytes.

(B) Elevated protein amounts of c-Fos and FRA1 in AdCre-*Adam17*$^{-/-}$ keratinocytes after PMA treatment.

(C) Enhanced AP-1 transcription factor binding for specific members of AP-1 family in AdCre-*Adam17*$^{-/-}$ keratinocytes treated with 10 ng/ml PMA for 3 hr.

(D and F) qPCR analysis of CSF-3, TSLP, and c-*fos* gene expression in keratinocytes 3 hr after PMA treatment. Presence of the pan AP-1 inhibitor Tanshinone IIA (TanIIA, D) or ectopic expression of active Notch (AdNICD, F) abrogates gene expression.

(E) Measurement of AP-1 luciferase reporter activity in MEFS after 8 hr of PMA treatment. Use of a specific c-Fos inhibitor (T-5224) or ectopic Notch (AdNICD) abolishes AP-1 luciferase activity.

*$p < 0.05$, mean ± SEM (n = 3). Data in (A)–(C) are representative of two independent experiments. Data in (D) and (E) are representative of three independent experiments.

keep epithelial cytokine production in check (outlined in Figure S6D).

DISCUSSION

We have shown that ADAM17 provides an inherent permissive signal for basal Notch activation throughout adult life. It triggers Notch signaling to regulate c-Fos activation, thereby maintaining epidermal barrier homeostasis. Loss of this control induces the production of cytokines or alarmins, which catalyze local and systemic immune responses even in the absence of an overt exogenous stress. Independent studies have shown that the loss of Notch, deregulation of AP-1, or overproduction of epithelial TSLP result in inflammatory skin disease (Demehri et al., 2009; Dumortier et al., 2010; Meixner et al., 2008; Zenz et al., 2005). The ADAM17-Notch-c-Fos triad uncovered in our study reveals a mechanistic underpinning of all these observations.

To date, studies of ADAM10-deficient mice have established its importance in Notch activation during development (Esteve et al., 2011; Gibb et al., 2010, 2011; Weber et al., 2011). On the other hand, the large majority of reported ADAM17-dependent phenotypes revolve around TNFR or EGFR signaling (Blobel, 2005; Guinea-Viniegra et al., 2009; Horiuchi et al., 2007;

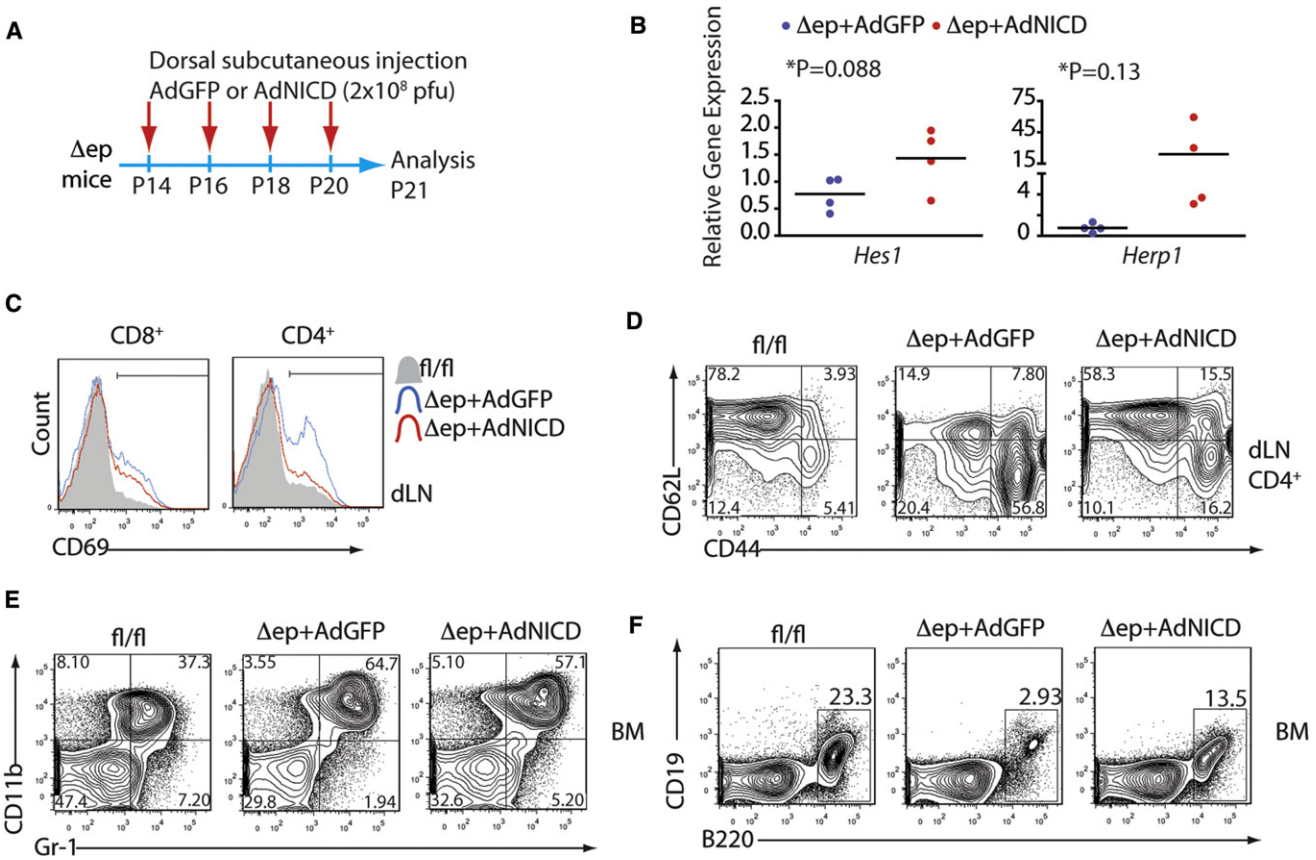

Figure 7. Ectopic Notch Activity Rescues Atopic Dermatitis and Myeloproliferative Disease in *Adam17*$^{\Delta ep}$ Mice

(A) Experimental protocol to reintroduce active Notch in dorsal skin of Δep mice by subcutaneous delivery of adenoviral vector harboring NICD (AdNICD).

(B) qPCR analysis of Notch target genes *Hes1* and *Herp1* in dorsal epidermis at experimental endpoint. Bars depict mean expression (n = 4).

(C and D) FACS analysis of dorsal dLNs to measure CD4$^+$ T cell activation by cell surface CD69 (C) and CD4$^+$CD62L$^-$CD44$^+$ effector T cells (D) in Δep mice treated with AdGFP or AdNICD.

(E and F) FACS analysis of bone marrow Gr-1$^+$CD11b$^+$ cells (E) and B220$^+$ cells (F) in Δep mice treated with AdGFP or AdNICD.

*$p < 0.05$ mean ± SEM (n = 4 for A, B; n = 3 for C–E). Data in (C)–(E) are representative of three independent experiments. Flow cytometry plots represent four mice per condition.

Maretzky et al., 2011; Mohammed et al., 2004; Murthy et al., 2010). Recent in vitro studies have brought to light the requirement of ADAM17 in ligand-independent Notch activation (Bozkulak and Weinmaster, 2009; van Tetering et al., 2009). Our study proposes that ligand-independent Notch signaling controls barrier immunity but not keratinocyte development. The developmental context of Notch activation may be fulfilled by ADAM10, because mice lacking epidermal *Adam10* fail to establish a spinous layer of the epidermis (Weber et al., 2011), whereas *Adam17*$^{\Delta ep}$ mice exhibit precocious keratinocyte differentiation. Epidermal homeostasis balances cell-autonomous keratinocyte development and nonautonomous communication between epithelial, stromal, and immune compartments in the skin. By generating inducible deletion of epidermal *Adam17* at 8 weeks of age, we have demonstrated the significance of ADAM17 in maintaining proper epithelial: immune crosstalk in the adult skin.

In addition to the established role of Notch activation in keratinocyte development and transformation, it is now accepted that Notch activation keeps the epidermal stress response in check (Nicolas et al., 2003; Williams et al., 2011). Accumulating evidence suggests that Notch promotes NF-κB signaling but inhibits AP-1 activity; both of these pathways are key components of stress signaling in keratinocytes but the molecular mechanism by which Notch and AP-1 pathways crosstalk have remained elusive (Chu and Bresnick, 2004; Chu et al., 2002; Meixner et al., 2008; Pasparakis et al., 2002; Zenz et al., 2005). Although we did not observe defects in NF-κB signaling (data not shown) or rescue AD through *Tnfr1* deletion, we elucidated how ADAM17-dependent Notch activation prevents spontaneous AP-1 activation and atopic immunity. Further, the data mining performed in this study indicated a clear deregulation of the Notch pathway in human atopic dermatitis. Supporting our findings, polymorphisms have been reported in patients where enhanced AP-1 recruitment to *Tslp* and *Il4* promoters occurs in several human atopic diseases (Harada et al., 2010; Pastore et al., 2000; Song et al., 1996). More recently, a clinical report has identified that loss-of-function mutations in human *Adam17* lead to spontaneous inflammatory skin disease with a similar etiology

to that of *Adam17*$^{\Delta ep}$ mice (Blaydon et al., 2011). Our study provides a mechanistic explanation for the development and progression of epidermal inflammation observed in humans.

It is important to note that additional Notch-dependent and -independent pathways are probably involved in regulating epithelial cytokine synthesis in this model. Although our findings present an antagonistic interplay between active Notch and c-Fos on cytokine promoters, it is known that the Notch target gene *Hes1* acts as a bona fide transcriptional repressor in epidermal development (Moriyama et al., 2008). Because we noted decreased *Hes1* gene expression in ADAM17-deficient epidermis and in primary keratinocytes, it is possible that a loss of *Hes1*-mediated suppression contributes to increased TSLP or G-CSF production. Finally, we and others show that c-Fos promotes its own transcription (Figure 6D). The loss of epidermal *Adam17* could thus result in a positive feedback loop wherein accumulating c-Fos transcribes itself, culminating in undesired epithelial cytokine synthesis.

Epithelial cells are now recognized as orchestrators of the peripheral immune response, and several recent studies have identified epithelial mutations causal to undesired immune cell activation in barrier tissues (Biton et al., 2011; Islam et al., 2011; Pasparakis, 2009; Swamy et al., 2010). We have demonstrated a gatekeeper function of ADAM17 in the skin, which may apply to other mucosal tissues including gut, lung, and vaginal epithelium. The observation that epidermal ADAM17 suppresses the accumulation of IL-17-producing lymphocytes (both $\gamma\delta$ T cells and Th17 T cells) has implications for the treatment of acute dermatitis, because rising IL-17 amounts strongly correlate with severity of AD in human patients (Di Cesare et al., 2008; He et al., 2007; Koga et al., 2008). The epimmunome continues to grow and understanding the mechanisms that instruct pro- or anti-inflammatory epithelial cytokine synthesis will be of value in the treatment of diseases afflicting barrier tissues.

EXPERIMENTAL PROCEDURES

Mice

Adam17$^{fl/fl}$ mice were generated by C.P. Blobel and backcrossed >9 times into the C57BL/6 background. *Krt14-cre* transgenic mice (Tg(*Krt14-cre*)1Amc/J) and *Tnfr1*$^{-/-}$ mice (C57BL/6-*Tnfrsf1a*^{tm1Imx}/J) were originally obtained from Jackson laboratories. *Krt14-cre* mice were crossed with wild-type C57BL/6 mice and maintained as single-hemizygous genotypes harboring a single *cre* allele. Epidermal *Adam17* deletion was created by generating Krt14-*cre; Adam17*$^{fl/fl}$ mice harboring a single *cre* allele. *Adam17*$^{fl/fl}$ mice were used as control genotypes as loss of a single *Adam17* allele (*Krt14cre;Adam17*$^{fl/+}$ mice) resulted in an intermediate phenotype (data not shown). For tamoxifen-induced deletion of *Adam17* in *Krt14-CreER;Adam17*$^{fl/fl}$ mice, tamoxifen (Sigma, T5648) was prepared by dissolving in sesame oil and repeated sonication, and administered intraperitoneally (i.p., 1 mg per in 400 μl oil) to 8-week-old mice for 5 consecutive days. As controls, *Adam17*$^{fl/fl}$ mice were treated with tamoxifen. Mice were sacrificed and analyzed 3 or 8 months after treatment. Mice were fed 5%-fat chow ad libitum and housed and cared for in accordance with protocols approved by the Canadian Council for Animal Care and the Animal Care Committee of the Ontario Cancer Institute.

In Vivo Delivery of Active Notch to Rescue Epidermal Inflammation

Mice aged 14 days were treated with a dorsal subcutaneous injection of 4× 10^9pfu/ml adenovirus encoding active Notch (AdNICD, kindly provided by E. Wagner, Centro Nacional de Investigaciones Oncologicas, Madrid, Spain) or eGFP (Ad-eGFP, Vector Biolabs) dissolved in 50 μl sterile PBS. This treatment was administered every 48 hr at P14, P16, P18, and P20. At P21, mice were sacrificed and tissue harvested for analysis. Notch signaling was confirmed by qPCR analysis of target genes *Hes1* and *Herp1* from dorsal epidermis scrapings. Epidermis was obtained by incubating minced dorsal skin in Liberase Blendzyme (Roche) for 1 hr at 37°C with periodic vortexing.

Isolation and Culture of Primary Keratinocytes

Mouse keratinocytes were obtained from *Adam17*$^{fl/fl}$ pups according to established protocols (Lichti et al., 2008). Cell-autonomous *Adam17* deletion was generated by transducing *Adam17*$^{fl/fl}$ keratinocytes with adenoviral Cre (AdCre-IRES-GFP, Vector Biolabs) or adenoviral GFP as control (Ad-eGFP, Vector Biolabs) at an MOI of 3. Notch overexpression was induced by transduction with AdNICD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures (reagents, microarray analysis, chromatin immunoprecipitations, and functional assays), six figures, and two tables and can be found with this article online at doi:10.1016/j.immuni.2012.01.005.

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